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The CXCR4 mutations in WHIM syndrome impair the stability of the T cell immunological synapse

Short title: **WHIM-mutant CXCR4 impairs T cell synapse stability**

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Key Point

- The CXCR4 mutations in the WHIM syndrome impair the stability of the T cell-Antigen Presenting Cell immunological synapse

The WHIM (Warts, Hypogammaglobulinemia, Infections and Myelokathexis) syndrome is a rare disease characterized by diverse symptoms indicative of aberrantly functioning immunity. It is caused by mutations in the chemokine receptor CXCR4, which impair its intracellular trafficking, leading to increased responsiveness to chemokine ligand and retention of neutrophils in bone marrow. Yet WHIM patient symptoms related to adaptive immunity, such as delayed IgG-switching and impaired memory B cell function, remain largely unexplained. We hypothesized that the WHIM-associated mutations in CXCR4 may affect the formation of immunological synapses between T cells and antigen presenting cells (APC). We show that, in the presence of competing external chemokine signals, the stability of T-APC conjugates from patients with WHIM-mutant CXCR4 is disrupted due to impaired recruitment of the mutant receptor to the immunological synapse. Using retrogenic mice that develop WHIM-mutant T cells, we show that WHIM-mutant CXCR4 inhibits the formation of long-lasting T-APC interactions in *ex vivo* lymph node slice time-lapse microscopy. These findings demonstrate that chemokine receptors can affect T-APC synapse stability and allow us to propose a novel mechanism that contributes to the adaptive immune response defects in WHIM patients.

Introduction

The rare human WHIM syndrome is characterized by neutropenia, myelokathexis, delayed antibody class switching to Immunoglobulin G (IgG)¹, long-term hypogammaglobulinemia and memory B cell lymphopenia,² recurring infections and human papillomavirus-induced warts.³ WHIM is associated with C-terminal, dominant mutations in the chemokine receptor CXCR4, resulting in truncation of the receptor.⁴ This leads to defective recycling upon binding of the CXCR4 ligand, CXCL12, and thus increased responsiveness to CXCL12 stimulation.^{2,5,6} The hyperfunctional CXCR4 mutations may thus explain neutrophil retention in the bone marrow (myelokathexis), where CXCL12 is expressed,⁷ and the consequent neutropenia in the periphery.⁸ However, the mechanism of pathogenesis of the remaining WHIM symptoms is less clear.³

Recurrent infections, the delay in production of IgG-switched antibodies found in one study¹ as well as the impaired memory B cell function and lack of long-term antibody titers following immunization,² are indicative of defects involving the adaptive immune response, required for effective and lasting defense against invading pathogens. The initiation of the adaptive response involves antigen-specific activation of T cells in the secondary lymphoid organs, where APC home to after taking up antigen in the periphery. Following successful activation via a T cell-APC immunological synapse formation, antigen-activated CD4⁺ T cells can then provide co-stimulatory signals to B cells, which enable the B cells to undergo immunoglobulin class switch recombination and thus synthesize IgG, IgA and IgE.⁹ The resultant memory B cells and plasma cells subsequently migrate to their niches.¹

It has been proposed that, in a manner analogous to the effect on neutrophils, WHIM-mutant CXCR4 on B cells may account for both the initial delay in mounting an IgG antibody response and the long-term memory B cell dysfunction, by affecting the homing of the B cells.¹

We have previously demonstrated that chemokine receptors, in addition to controlling leukocyte homing and migration, may increase the stability of the T cell-APC immunological synapses,¹⁰ the specialized junctions that form at the initiation of adaptive immune responses and which are necessary for successful T cell activation.^{11,12} Indeed, *in vitro* studies have led to the hypothesis that chemokines in lymph nodes may either enhance the stability of the synapse -by reinforcing the “stop” signal transduced by the antigen-triggered T cell receptor (TCR)- or inhibit T-APC interactions -by providing “go” signals overcoming the TCR-.¹³⁻¹⁵ Whether this regulates T cell activation *in vivo* is still unknown.

Given the hyperfunctional nature of WHIM-mutant CXCR4,^{2,5} we hypothesized that the WHIM-associated mutations in CXCR4 may interfere with the robustness of the T cell-APC synapses. This would be expected to affect T cell activation, as well as the B cell functions that are dependent on T cell activation, such as immunoglobulin class switching. Here we show that the WHIM-associated mutations of CXCR4 impair T-APC synapse stability and may be affecting T cell priming, while causing a delay in IgG responses. These findings identify a novel mechanistic explanation for the defects in the early stages of adaptive immune responses in the WHIM syndrome, whilst also offering a novel and natural demonstration of the role of chemokines in the regulation of the immunological synapse stability.

Methods

Retrogenic mice

Animals were kept in an SPF facility and treated according to Institutional and National guidelines and regulations. Retrogenic mice were created as in¹⁶: bone marrows (BM) were harvested from donor OT-II ovalbumin (OVA)-specific TCR-transgenic CD45.1 mice, that had been pre-treated with 5-fluoracil at 5.5mg/25g mouse, 4 days prior to harvesting. Harvested BM cells were grown in 20ng/ml rmIL-3, 50ng/ml rmIL-6 and 50ng/ml rmSCF (all R&D Systems) for 48h and then transduced with retroviral vectors for EGFP-WT-CXCR4 or EGFP-WHIM-CXCR4 and pCLEco (Imgenex) on day 2, 3 and 4 post-BM harvesting, using fresh viral supernatants, as in¹⁷. The transduced BM cells were kept in culture for a further 2 days prior to intravenous injection into irradiated (2x475 Rad) C57BL/6 recipients. These recipients were used as retrogenic mice, 7 weeks post-intravenous injection.

T cell purification

Patient and healthy donor samples were obtained after informed consent at Clinica Pediatrica and Humanitas, according to the institutional ethical committee guidelines and the Declaration of Helsinki. Primary human CD4⁺ T cells from peripheral blood were isolated using RosetteSep (StemCell Technologies), at a typical purity of >90%. CD4⁺ T cells were transduced with CXCR4 constructs using Amaxa human T cell nucleofactor (Lonza). For experiments with patient samples, PBMC from WHIM patients and healthy age/gender-matched donors were expanded for 2 weeks after activation with 2.5 µg/ml PHA (Biochrom AG), 400 U/ml IL-2 (Chiron) and irradiated feeders; CD4⁺ T cells were then isolated using a FACSaria (BD) cell

sorter. Mouse CD4⁺ T cells from retrogenic mice were harvested at 7 weeks after bone marrow transfer, purified using MACS CD4⁺ kit (Miltenyi Biotec) and then sorted for EGFP expression on FACSaria. For lymph node slice time-lapse 2-photon imaging experiments, the CD4⁺ EGFP⁺ WT or WHIM-mutant CXCR4-expressing retrogenic cells were labeled with 2μM CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) (Invitrogen). EGFP⁻ non-retrogenic T cells were labeled with 10μM Blue-CMAC (7-Amino-4-Chloromethylcoumarin) (Invitrogen) and used as a control population. Changing the dye used in the experimental populations did not modify the result. Nonetheless, as an additional measure, we chose to use the same dye for the 2 experimental populations, in order to ensure that no differential toxic effects of the two dyes could affect measurements. As this necessitated imaging of the 2 populations in consecutive slices, the same Blue-CMAC-labeled, non-retrogenic control population was used as an internal control in both cases.

T cell-APC conjugates

The conjugate stability assay by transwell migration and the analysis of receptor recruitment to the synapse were adapted from¹⁰, with the following modifications: conjugates were formed between WT or WHIM-mutant CXCR4-expressing CD4⁺ T and primary B cells obtained via RosetteSep (StemCell Technologies). B cells had been pulsed with superantigen (1μg/ml staphylococcal enterotoxin A/B/E, Toxin Technology) and labeled with PKH26 dye (Sigma) and were used at a 1:1 ratio with T cells. Cells were then allowed to migrate through 3μm pores to 2.5nM CXCL12 (R&D Systems) for 2h, before analyzing by FACS. Conjugates prepared for confocal microscope analysis of receptor recruitment were made using EBV-B as APC, and allowed to form in the presence or absence of CXCL12 (5nM-100nM) and AMD3100

(Sigma, 12.6 μ M). Relative Recruitment Index (RRI) for EGFP-CXCR4 was calculated as in¹⁰.

Two-photon microscopy imaging

Lymph node slice preparation was adapted from¹⁸. Bone marrow-derived dendritic cells (DC) were pulsed with 10 μ g/ml OVA(323-339)(Anaspec), labeled with 5 μ M CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) dye (Invitrogen) and injected along with 40ng LPS (Sigma) at 4X10⁶ cells/mouse subcutaneously into C57BL/6 mice. The following day the recipients were sacrificed and inguinal lymph nodes were extracted, enclosed in agarose and sliced using a vibratome (Leica VT 1000S). Labeled T cells from retrogenic mice were allowed to enter the lymph node for 1h prior to 3-colour time-lapse imaging for 30 minutes with a 30 second inter-frame interval on a LaVision TrimScope 2-photon microscope. As in¹⁸, the lymph node slices were placed in AMD3100 (Sigma, 12.6 μ M); to avoid the reported inhibition of entry into the lymph node slice, T cells were allowed 30 min in AMD3100-free medium to enter to the lymph node, followed by 30 min incubation in the presence of the inhibitor. AMD3100 was also present in the perfusion medium. Image analysis was performed on Imaris (Bitplane), using manually-corrected automatic tracking, and manual calculation of T-DC interaction times.

Activation experiments

T cell priming was performed by injecting 500ng OVA peptide in CFA into each hind footpad of WT or WHIM retrogenic mice. Draining lymph nodes and spleen were analyzed by FACS 12h after injection, for the expression of EGFP and staining for CD45.1 (A20; eBiosciences), CD4 (GK1.5; BioLegend) and CD69 (H1.2F3; BD).

Immunizations for IgG production experiments were performed as in¹⁹, using 100µg/mouse of NP(4-hydroxy-3-nitrophenylacetyl)-OVA (Biosearch) in Imject Alum (Pierce). ELISA assays for the detection of anti-NP IgG were adapted from²⁰, coating plates with 10µg/ml NP-BSA (Biosearch, Ratio>20) and detecting with Anti-mouse IgG-Peroxidase (Sigma). Human primary T cell activation by anti-CD3/anti-CD28 and IFNγ detection by ELISA was performed as in¹⁰. For mouse in vitro APC-free T cell activation, 2µg/ml anti-CD3e (145-2C11; BD) and 1µg/ml anti-CD28 (37.51; BD) were used for coating flat-bottom 96 well plates. The activation of rgWT and rgWHIM T cells by DC (at a 2:1 ratio) in the presence or absence of 25nM CXCL12 in the lower chamber of transwell assay plates was analyzed by FACS after staining for CD25 (PC61.5; eBiosciences).

Results

WHIM-mutant CXCR4 disrupts the stability of T cell-APC conjugates in WHIM patients

Given the association of the WHIM-mutant CXCR4 with enhanced chemotactic properties,^{2,5} we decided to assess whether the mutant chemokine receptor could impair the stability of the T-APC interaction in WHIM patients. Peripheral blood CD4⁺ T cells purified from a WHIM patient (G336X) or a healthy age- and gender-matched donor were used in conjugate stability experiments.¹⁰ T cells were allowed to form conjugates with labeled, superantigen-pulsed primary B cells. Non-pulsed B cells, which cannot form successful T-B conjugates, were used as controls. The conjugates were then allowed to migrate in a transwell assay towards CXCL12, using a transwell filter pore size allowing only single cells to pass through. The number of migrated CD4⁺ T cells is thus an indication of lack of conjugate stability.¹⁰ As expected, T cells from healthy donors were significantly impaired from migrating to CXCL12 when superantigen-pulsed B cells were used for conjugate formation, suggesting that “go” signals delivered by wild-type (WT) CXCR4 are subordinate to the “stop” signal delivered by the antigen-triggered TCRs (Figure 1A, left panel), in agreement with previous *in vitro* studies.¹³ However, under the same conditions, the chemotactic response of T cells from WHIM patients was strong and undiminished by the presence of antigen-loaded APCs (Figure 1A, right panel). The values shown are normalized to enable comparison between different donors. Thus, WHIM patient CD4⁺ T cells exhibit impaired conjugate stability in the presence of competing external CXCL12 signals.

Expression of WHIM-mutant CXCR4 disrupts the stability of T cell-APC conjugates in healthy donor cells

To prove that this is indeed due to the mutant CXCR4, we cloned the WT and WHIM-mutant (R334X) versions of CXCR4 fused with Enhanced Green Fluorescent Protein (EGFP). The ectopically expressed receptor was functional, as it could lead to phosphorylation of MAP kinases after CXCL12 administration (data not shown). Importantly, the WHIM-mutant CXCR4 was refractive to ligand-induced internalization (supplemental Figure 1), which is a hallmark of the WHIM syndrome.^{5,6} We expressed WT or WHIM-mutant CXCR4 in primary CD4⁺ T cells from healthy donors and used them in conjugate stability experiments, as above. As the WHIM mutation is dominant,³ endogenous WT CXCR4 expression in transfected cells is unlikely to interfere with the experimental outcome. CXCL12 was able to break conjugates formed with T cells expressing the WHIM-mutant but not with WT CXCR4 (Figure 1B). This demonstrates that the WHIM-mutant CXCR4 *per se* has destabilizing effects on the immunological synapse when exposed to competing cognate chemokine. As CXCL12 is present in the lymph node environment²¹ this “distracting” effect could impact on physiological T cell activation.

WHIM-mutant CXCR4 affects T cell-APC long-lasting interactions

To assess the physiological relevance of our findings, we tested our hypothesis utilizing *ex vivo* organ cultures of mouse lymph node slices.^{18,22} As human CXCR4 is functional in mice,⁸ we created retroviral versions of the CXCR4 constructs described above and used them to make retrogenic mice¹⁶ for WT or WHIM-mutant CXCR4. These mice generate mature, unactivated ovalbumin(OVA)-antigen-specific T cells expressing WT or WHIM-mutant CXCR4, 6 weeks after reconstitution of irradiated

recipients with transduced bone marrow cells^{16,23} (see Methods).

The WHIM retrogenic mice showed a 4-fold increase in bone marrow retention of WHIM-mutant CXCR4-expressing leukocytes in sentinel animals (data not shown), similar to previously reported models.⁸ Nonetheless, the mice ought to be considered as “retrogenic cell generators” rather than disease models. Indeed, our intention was to analyze the effects of the WHIM mutations on T cell-mediated responses to antigenic stimulation, in a physiological environment but in the absence of additional, complicating variables that may be affected by WHIM-mutant CXCR4 expression on other tissues.

From the retrogenic mice, we purified CD4⁺ T cells expressing retrogenic WT (rgWT) or WHIM-mutant (rgWHIM) CXCR4. OVA-specific T cells with no retrogenic expression were used as a control population. The T cells were labeled and allowed to enter into *ex vivo* organ cultures of lymph node slices,^{18,22} which had been extracted from mice that had received OVA antigen-pulsed, CMTMR-labeled dendritic cells (DC), on the previous day. The slices were imaged using 2-photon time-lapse video microscopy, starting 1 hour after administration of the T cell populations onto the lymph node slices. We analyzed all T cells that interacted with DC during the 30 minutes of each video. Control T cells, with no retrogene expression, formed more long-lasting interactions (clustering at ≥ 20 min) in the presence of OVA-pulsed as opposed to unpulsed DC (supplemental Figure 2), in agreement with previous reports.²⁴ The difference in the number of long versus short interactions was significant (Figure 2A). T cells expressing rgWT CXCR4 exhibited an identical behavior (Figure 2A and Video 1-3), indicating that, in this assay, the retrogenic expression *per se* does not distort the physiological function of the cells. Interestingly, rgWHIM T cells did not show any significant increase in the number of

long-lasting interactions (Figure 2A; also plotted as % in Figure 2B; black bars; Video 4-5) with antigen presence, thus confirming that the WHIM mutation impairs the ability of T cells to form long-lasting interactions with antigen-pulsed DC. In the presence of cognate antigen-pulsed DC the mean 3D speed of the WT cells was 1.7 $\mu\text{m}/\text{min}$ versus 2.3 $\mu\text{m}/\text{min}$ for the WHIM T cells, which is consistent with the more frequent interactions of the latter with DC.

As the observed impairment could be due to defective development of the rgWHIM T cells, we repeated the above experiments in the presence of AMD3100, a CXCR4 inhibitor that is identical to FDA and EMEA approved drug plerixafor.²⁵ In the presence of AMD3100, rgWHIM cells no longer differed from rgWT cells in the proportion of long-lasting interactions (Figure 3A; also plotted as % in Figure 3B; Video 6). Thus, the defective T-DC conjugate stability of rgWHIM cells was reversible within the lymph node slice and is therefore unlikely to be caused by upstream developmental defects. Although at very high concentrations, not achievable in vivo with the maximum FDA-approved dose, AMD3100 may act as a CXCR7 agonist,²⁶ the complete reversal of the phenotype caused by the mutant CXCR4 suggests that, in this context, AMD3100 does indeed act as a CXCR4 inhibitor. Cells expressing WT CXCR4 were expectedly unaffected, as it has been shown that WT CXCR4 signaling within the lymph node microenvironment is inconsequential for T cell activation.^{18,27}

The recruitment of WHIM-mutant CXCR4 to the Immunological Synapse is disrupted by competing chemokine signals

In order to gain insight on the mechanism that underlies the disruption of synapse stability by WHIM-mutant CXCR4, we examined the cellular distribution of the

receptor during T-APC conjugate formation. We expressed the EGFP-fused WT or WHIM-mutant CXCR4 in primary CD4⁺ T cells from healthy donors that were allowed to form conjugates with superantigen-pulsed EBV-B cells, in the presence of competing external chemokine ligand at a wide range of concentrations. We then analyzed the recruitment of the fluorescent CXCR4 molecules to the T-APC synapse by confocal microscopy. WT CXCR4 (Figure 4A, white bars) was significantly recruited to the synapse when superantigen-pulsed EBV-B cells were used, as previously shown.¹⁰ This recruitment was not significantly reduced by the presence of competing, external CXCL12 ligand in the culture medium, a finding that is compatible with the attribution of lack of “dominance” over TCR signals to WT CXCR4-CXCL12 interactions.¹⁴ Interestingly, whilst WHIM-mutant CXCR4 (Figure 4A, black bars) was also significantly recruited to the synapse in the presence of superantigen, this recruitment was significantly reduced in the presence of competing CXCL12, suggesting a possible mechanistic explanation for the disruptive effect of the mutant receptor on immunological synapse stability. Furthermore, mirroring our *ex vivo* findings, concurrent presence of AMD3100 as well as CXCL12 in the culture medium was able to fully reverse the loss of mutant receptor recruitment to the synapse.

WHIM-mutant CXCR4 leads to aberrant *in vivo* T cell priming and a delayed IgG response

Even though not all stable T-APC conjugates would be expected to lead to the initiation of an immune response, a significant impairment in the stability of immunological synapses is likely to cause aberrant T cell activation *in vivo*.^{11,12} To test this in our system, we injected OVA peptide into the footpads of WHIM-mutant

or WT CXCR4 retrogenic mice and examined their EGFP-expressing, retrogenic T cells in draining lymph nodes, 12h after antigen administration. rgWT T cells displayed moderately yet significantly higher expression of the early T cell activation marker CD69²⁸ than rgWHIM T cells (Figure 5A, right panel and 5B). In non-draining lymphoid compartments, such as the spleen (Figure 5A, left panel), no difference was observed. These results suggest that WHIM-mutant CXCR4-expressing T cells exhibit aberrant T cell priming. To understand whether this result may depend on intrinsic defects of the TCR/CD3 complex of rgWHIM T cells, we performed in vitro activation experiments using anti-CD3 and anti-CD28 antibodies. T cells from WHIM-mutant retrogenic mice, as well as WHIM patient (G336X) T cells, displayed fully functional TCR signalling (Figure 5C-D). Then, we tried to reproduce in vitro the conditions present during T cell activation in lymph nodes that may affect the stability of the synapse. T cells were stimulated with peptide-pulsed DCs in a transwell chamber (upper well), in the presence or in the absence of the “distracting” effects of CXCL12 (lower well). When CXCL12 was present in the lower chamber, rgWHIM but not rgWT T cells displayed a modest, although significant, reduction in activation (Figure 5E-F).

To confirm the physiological relevance of our findings, we assessed whether the aberrant T cell priming could lead to T cell-dependent early activation defects that characterize WHIM syndrome patients, such as the delay in IgG responses found in one study.¹ To this end, we transferred 25×10^3 OVA-specific rgWT or rgWHIM CD4⁺ T cells into RAG1^{-/-} mice, which are devoid of functional T and B cells. To provide antibody-producing cells, we co-transferred 6×10^6 T-depleted lymphocytes from WT syngenic donors. The recipient mice were immunized intraperitoneally with

OVA coupled to the hapten NP. WT B cells that can recognize NP²⁹ will take up NP-OVA and present peptides from both NP and OVA to T cells. Co-stimulation of the B cells by the transferred OVA-specific retrogenic T cells, which will represent the vast majority of the T cells present, will enable B cells to undergo class switch recombination and produce IgG antibodies against NP. As expected from previous studies,²⁹ by day 7 after immunization, recipients of rgWT T cells displayed a significant increase of anti-NP IgG in their sera (Figure 6, grey bars). However, this failed to occur in recipients of rgWHIM T cells, which did not mount a significant IgG response (Figure 6, black bars), remaining at levels found in T cell-depleted controls (data not shown). Yet by day 14 both groups of recipients displayed high IgG titers (Figure 6, day 14) and remained not significantly different in subsequent timepoints up to 6 months (data not shown). Thus the expression of WHIM-mutant CXCR4 on T cells leads to a delay in IgG responses, mirroring the delayed IgG switching observed in WHIM.¹

Discussion

The WHIM syndrome is characterized by symptoms affecting different stages of the adaptive immune response, the etiology of which is not clearly understood. Deficiencies in the late stages of adaptive responses include the memory B cell lymphopenia, inability to maintain long-term IgG antibody titers after immunization² and recurring infections.³ It has been proposed¹ that these defects may be caused, in similarity to the aberrant neutrophil homing, by impaired memory lymphocyte homing, a hypothesis supported by the panleukopenia in peripheral blood of WHIM patients.^{2,25} On the other hand, the delay in mounting IgG antibody responses observed in a patient studied by Mc Guire and colleagues¹ involves a T cell-dependent, B cell-mediated process that occurs at the early stages of the adaptive response. Our study offers a novel mechanistic explanation, at the heart of adaptive immune response initiation, for this observation. We demonstrate that immunological synapse stability is impaired in the presence of WHIM-mutant CXCR4. It is tempting to speculate that this is due to the lack of mutant receptor recruitment to the synapse, which, combined with the hyperfunctional nature^{2,5} of WHIM-mutant CXCR4 and the presence of CXCL12 in the lymph node,²¹ disrupts the TCR-mediated, synapse-promoting signals. This does not preclude the parallel existence of additional mechanisms, such as the proposed CXCR4-mediated aberrations in lymph node architecture,¹ which are in agreement with recent data from a mouse model of WHIM,³⁰ even though studies of hypogammaglobulinemia have yet to be performed in the latter.³¹ Given the wide expression range of CXCR4, it is likely that the combined effect of the above mechanisms, including the defect in synapse stability described here, contribute to the complex symptoms of the WHIM syndrome.

In the physiological milieu where T-APC immunological synapses form, “dominant” chemokine signaling has been proposed to inhibit stable synapse formation, based on results of *in vitro* experimentation.^{14,15} CXCL12 interacting with T cells expressing WT CXCR4 was found not to be a “dominant” chemokine.¹³ Rather, CXCR4 is recruited to the immunological synapse itself, where it enhances the activation of the T cell after receiving chemokine ligand produced by the APC.¹⁰ Exogenous chemokine signals that are not derived from the APC but from the surrounding lymph node microenvironment, where the chemokine is present,²¹ indeed did not affect T-APC synapse formation in WT CXCR4-expressing cells in our studies, in agreement with results showing lack of a role for WT CXCR4-CXCL12 on T cell activation.^{18,27} Yet our *in vitro* and *ex vivo* results show that WHIM-mutant CXCR4 enables CXCL12 to act as a distracting, “dominant” chemokine. This appears to occur via the disruption of the recruitment of WHIM-mutant CXCR4 to the synapse. Both the disruption of the receptor recruitment to the synapse and the “distracting” of stable synapse formation appear to be reversible by AMD3100 treatment, which has recently been proposed as a therapeutic agent for the WHIM syndrome.²⁵ Importantly, our findings also provide the first physiological, *ex vivo* evidence that chemokines and their receptors may control the stability of T-DC interactions in lymph nodes. The data suggest the existence of a delicate equilibrium between adhesive and chemoattractant forces operating in lymph nodes during T cell priming, allowing enough motility for T cell repertoire scanning whilst ensuring the formation of long-lasting conjugates, once a cognate T-APC pair is formed. Variations in this equilibrium, such as those due to the hyperfunctional mutant CXCR4 of WHIM

patients³ may lead to impaired stability of the immunological synapse and consequently contribute to an aberrant adaptive immune response.

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Contribution

MK, AS and AV designed the experiments. MK, AET and GR performed the experiments and analyzed the data. FA performed the 2PM imaging. LT and RB prepared the initial constructs and provided patient samples. MK and AV wrote the paper.

Conflicts of Interest Disclosure: The authors have no conflicting financial interests.

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Figure Legends

Figure 1. WHIM-mutant CXCR4 impairs T cell-B cell conjugate stability.

(A) Primary CD4⁺ T cells from healthy WT donor or WHIM-patient peripheral blood formed conjugates for 15' with superantigen (sAg)-pulsed, labeled primary B cells from a healthy allogenic donor. The conjugates were allowed to migrate towards CXCL12 in a transwell filter allowing the migration of single cells only. The migrated T cells were analyzed by FACS and the number of T cells migrated for each condition is shown. *, P < 0.05 (1-way ANOVA and Tukey's post test). Experiment performed twice, summary of results (mean +/- SEM) shown. (B) Primary CD4⁺ T cells from healthy donor peripheral blood transfected with EGFP-CXCR4 (WT) or EGFP-CXCR4 (WHIM) formed conjugates for 15' with sAg-pulsed primary B cells from the same donor. The conjugates were allowed to migrate towards CXCL12 in a transwell filter allowing the migration of single cells only. Migrated EGFP⁺ T cells were analyzed as above. **, P < 0.01 (1-way ANOVA and Tukey's post test). Experiment performed twice, results (mean +/- SEM) from representative experiment shown.

Figure 2. WHIM-mutant CXCR4 impairs T-DC interactions in *ex vivo* lymph node slice cultures.

See also Videos 1-5. DC were either cognate antigen-pulsed (OVA) or unpulsed (no pept). ctrl: non-retrogenic OVA-specific T cells. WT: OVA-specific T cells retrogenically expressing WT CXCR4. WHIM: OVA-specific T cells retrogenically expressing WHIM-mutant CXCR4. (A) Duration of T-DC interactions within *ex vivo* lymph node slices imaged by 2-photon microscopy. Summary results of 2 videos per condition shown. Each dot represents a single T-DC interaction. (from

left to right, n=125, 101, 66, 21, 298, 291, 197 and 183). An independent experiment with the T cell populations labeled with different dyes yielded the same result. A Fisher's exact test was applied to the number of interactions greater or equal to 20 min versus the number of interactions shorter than 20 min. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) The percentage of T-DC interactions greater or equal to 20 min, out of all analyzed interactions. White bars: non-retrogenic OVA-specific control T cells. Grey bars: OVA-specific T cells retrogenically expressing WT CXCR4. Black bars: OVA-specific T cells retrogenically expressing WHIM-mutant CXCR4. The statistical analysis refers to the Fisher's exact test, as in (A).

Figure 3. WHIM-mutant CXCR4-mediated impairment of T-DC interactions in *ex vivo* lymph node slice cultures is reversed by the CXCR4 inhibitor AMD3100.

See also Video 6. DC were either cognate antigen-pulsed (OVA) or unpulsed (no pept). ctrl: non-retrogenic control OVA-specific T cells. WT: OVA-specific T cells retrogenically expressing WT CXCR4. WHIM: OVA-specific T cells retrogenically expressing WHIM-mutant CXCR4. (A) Duration of T-DC interactions within *ex vivo* lymph node slices imaged by 2-photon microscopy, in the presence of 12.6 μ M AMD3100. Summary results of 2 videos per condition shown. Each dot represents a single T-DC interaction (from left to right, n=150, 103, 167, 158, 152, 301, 137 and 178). A Fisher's exact test was applied to the number of interactions greater or equal to 20 min versus the number of interactions shorter than 20 min. *, $P < 0.05$; ***, $P < 0.001$. (B) The percentage of T-DC interactions greater or equal to 20 min, out of all analyzed interactions. White bars: non-retrogenic OVA-specific control T cells. Grey bars: OVA-specific T cells retrogenically expressing WT CXCR4. Black bars: OVA-

specific T cells retrogenically expressing WHIM-mutant CXCR4. The statistical analysis refers to the Fisher's exact test, as in (B).

Figure 4. WHIM-mutant CXCR4 recruitment to the T-APC synapse is impaired by competing external CXCL12 and restored by AMD3100. (A) Primary CD4⁺ T cells from healthy donor peripheral blood transfected with EGFP-CXCR4 (WT) or EGFP-CXCR4 (WHIM) formed conjugates for 15' with unpulsed or superantigen (sAg)-pulsed EBV-B cells, in the presence or absence of 5nM to 100nM CXCL12 and 12.6μM AMD3100. The cells were fixed, imaged in confocal microscopy, and the Relative Recruitment Index to the T-APC synapse (RRI) for EGFP-CXCR4 was calculated. The graph displays the percentage of cells with CXCR4 recruited to the synapse (i.e. with RRI above the mean RRI of control WT T cells incubated with unpulsed EBV-B). The RRI data set for all analyzed cells (n≥40 per condition) is shown in (supplemental Figure 3). Statistical analysis was performed on the proportion of cells displaying recruitment versus non-recruitment of CXCR4 to the synapse. *, P < 0.05, ***, P < 0.001 (Fisher's exact test). (B) Representative confocal microscopy images showing the recruitment of EGFP-CXCR4 (WT) or EGFP-CXCR4 (WHIM) to the T-APC synapse.

Figure 5. Aberrant responses of WHIM-CXCR4 T cells due to reduced synapse stability. (A) WT or WHIM-CXCR4 retrogenic mice were immunized with OVA peptide in their hind footpads. Expression of early activation marker CD69 (anti-CD69 antibody Mean Fluorescence Intensity) on retrogenic CD4⁺ T cells from the spleen or draining lymph nodes (popliteal) of immunized animals (n=5) at 12h post-immunization is shown. A normality test was performed followed by an unpaired t-

test: Popliteal $P = 0.0015$; spleen $P = 0.9$. (B) Representative histograms of CD69 expression by EGFP⁺ CD4⁺CD45.1⁺ rgWT or rgWHIM T cells in the popliteal lymph nodes of the animals analyzed. (C) Healthy donor or WHIM patient (G336X) CD4⁺ T cells were activated by anti-CD3 and anti-CD28 coated beads. IFN γ production was measured by ELISA at 48h. Normalized results are shown to enable comparison of different donors. (D) rgWT or rgWHIM T cells were activated in tissue culture plates by anti-CD3 and anti-CD28. CD69 expression was measured at 12h. Anti-CD69 antibody Mean Fluorescence Intensity on CD4⁺EGFP⁺ rgWT or rgWHIM T cells is shown. **, $P < 0.01$; ***, $P < 0.001$ (1-way ANOVA and Tukey's post test). Representative experiment of two experiments shown. (E) rgWT or rgWHIM T cells were placed with cognate antigen-pulsed (OVA) DC in the upper chamber of a transwell assay plate, whilst 25nM CXCL12 was added or not to the lower chamber. The cells were incubated for 2h in the transwell plates before being transferred to serum-containing wells for 12h. The number of CD25⁺ cells among EGFP⁺CD4⁺ T cells in pooled upper and lower chamber cells at the end of the incubation is shown in (E), normalized for differences in the transduction efficiency between the rgWT and rgWHIM donor mice. CD25 expression (anti-CD25 antibody Mean Fluorescence intensity) on EGFP⁺CD4⁺ T cells of pooled upper and lower chamber cells at the end of the incubation is shown in (F). For both *, $P < 0.05$ (repeated measures 1-way ANOVA). Experiment performed twice, summary of results (mean \pm SEM) shown. It is noteworthy that, in the absence of antigen, the rgWHIM T cells displayed a higher baseline CD25 expression.

Figure 6. WHIM-CXCR4 expressing retrogenic T cells lead to a delay in IgG antisera production. RAG1^{-/-} recipient mice (n=5 per group) were reconstituted with

retrogenic WT (rgWT) or WHIM (rgWHIM) T cells specific for OVA as well as T-depleted splenocytes, and immunized with NP-OVA. The bars show anti-NP IgG titers in the sera at various timepoints after immunization. **, $P < 0.01$, ***, $P < 0.001$ (2-way ANOVA and Bonferroni post tests).

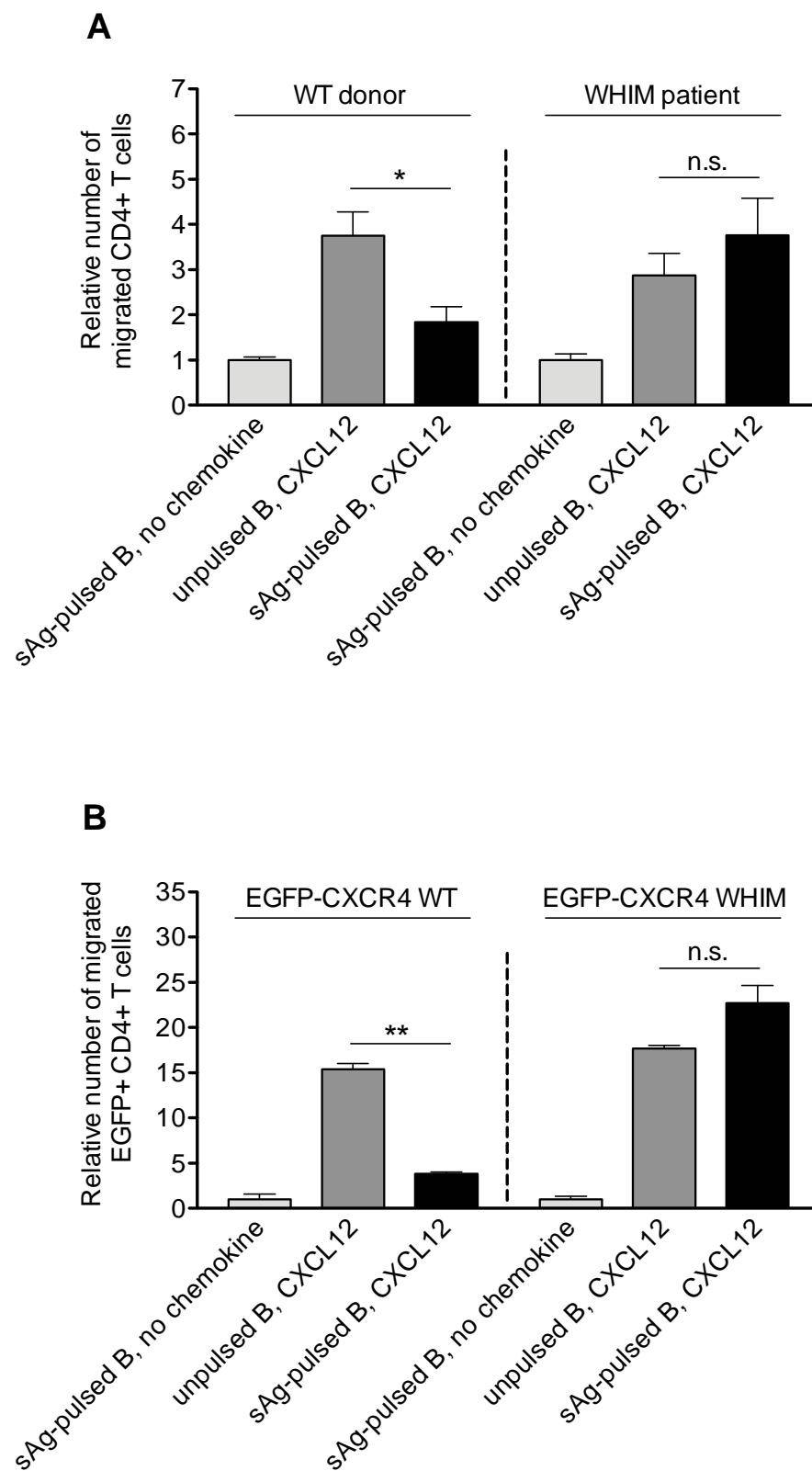


Figure 1

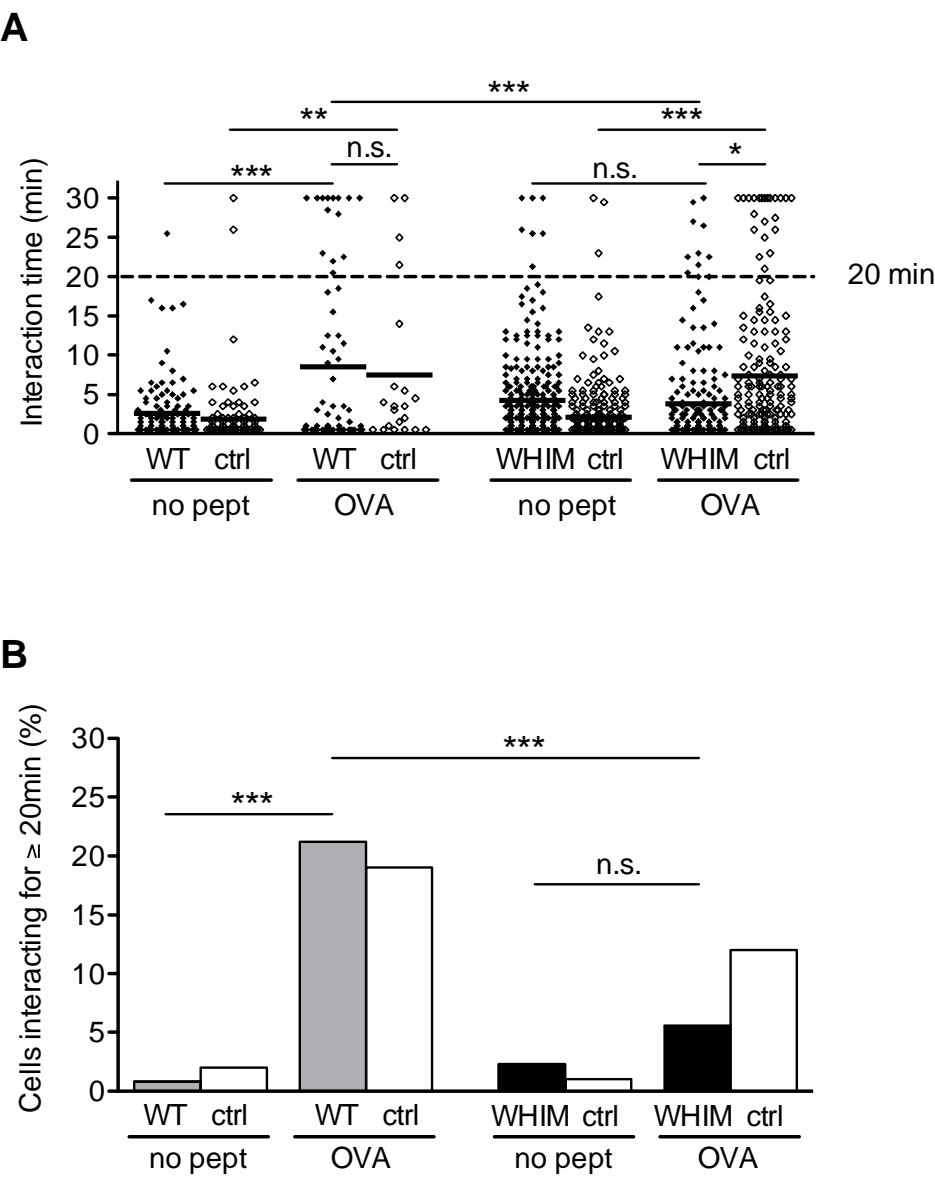


Figure 2

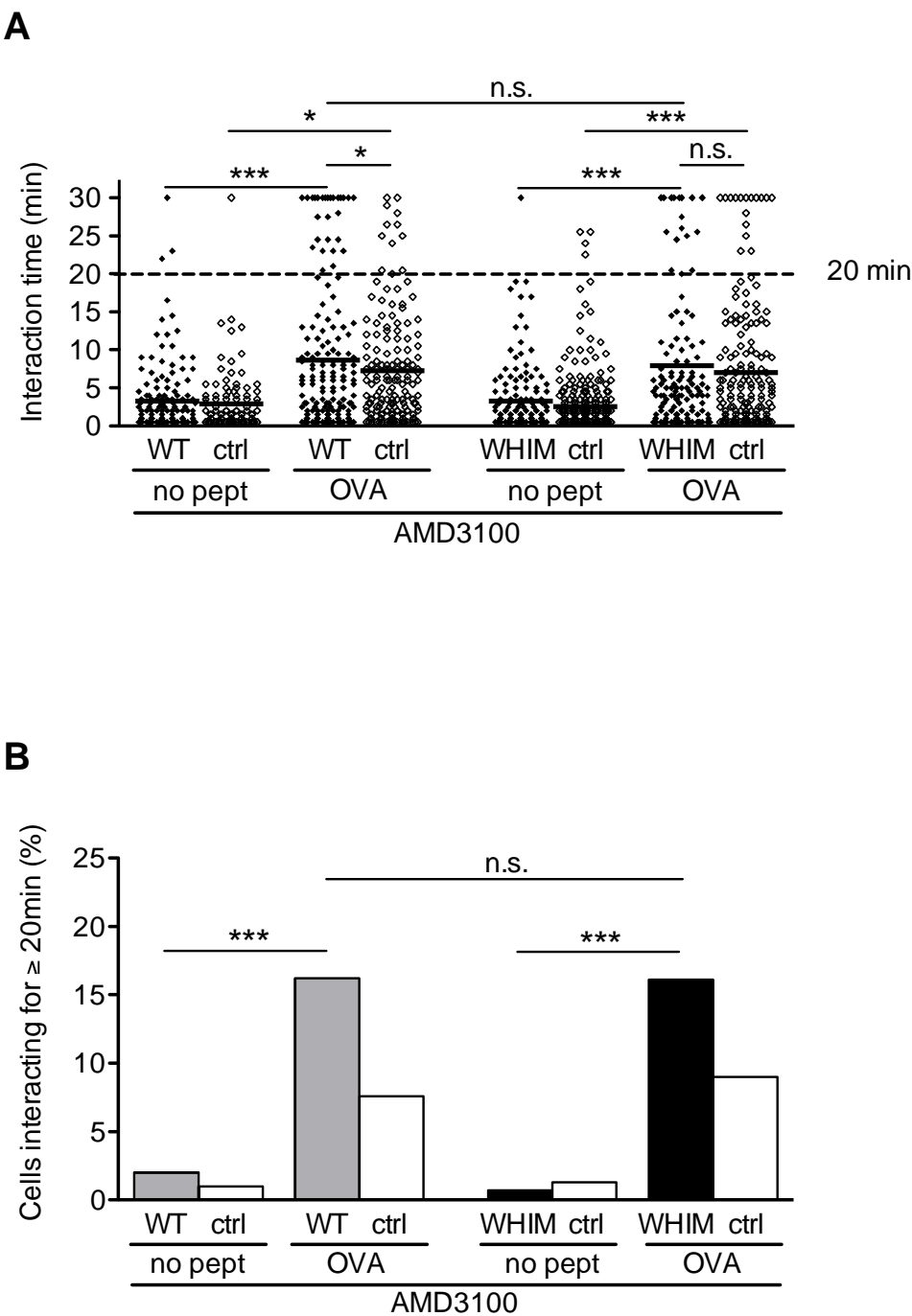


Figure 3

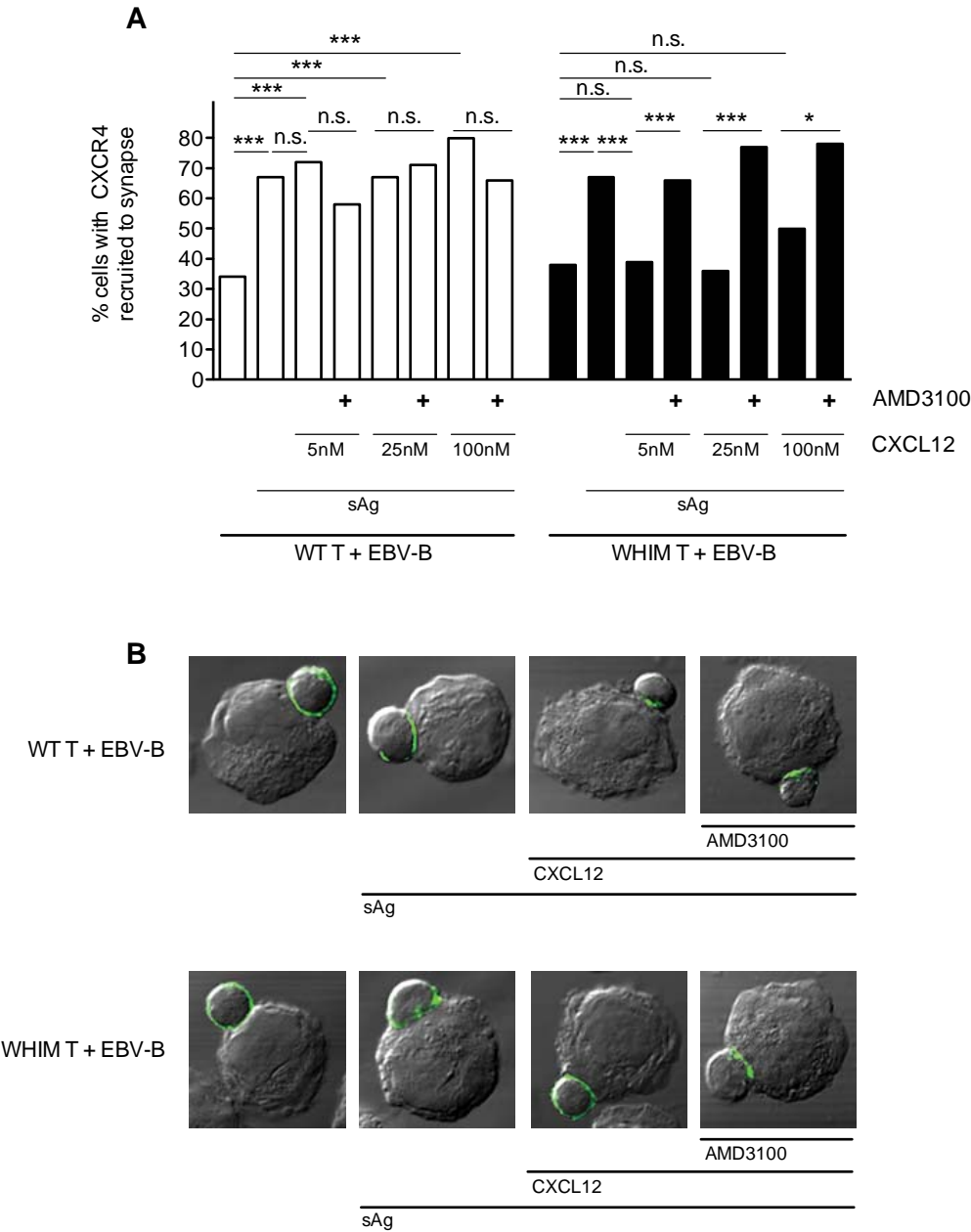


Figure 4

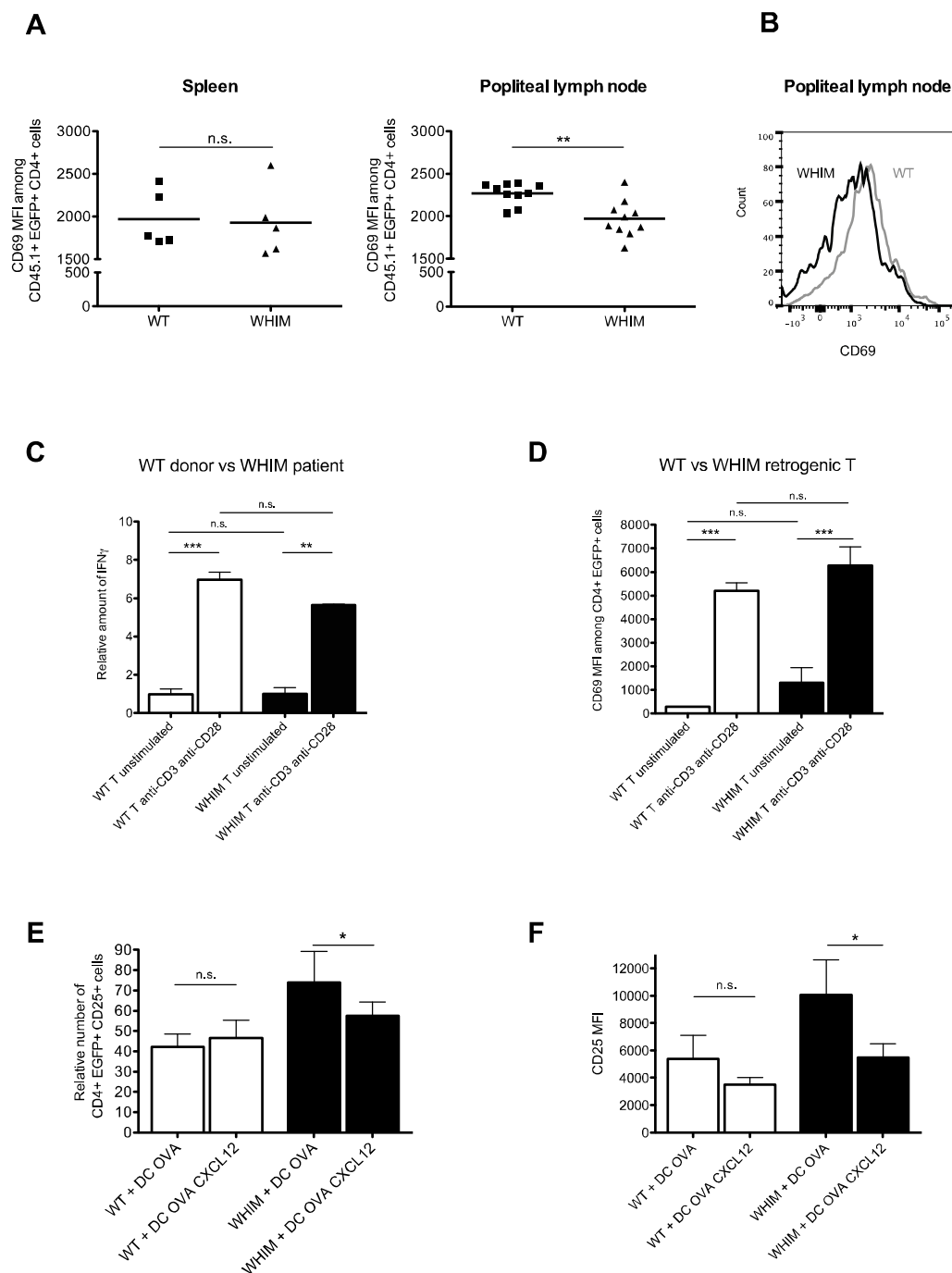


Figure 5

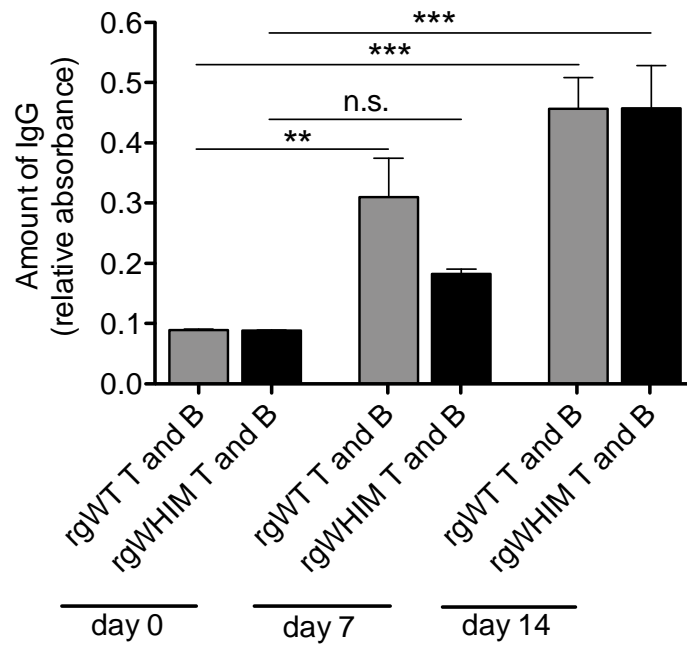


Figure 6